CHARACTERIZATION OF THE LARGE FRAGMENTS FROM PEPSIN-DIGESTED, DENATURED COLLAGEN

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SUMMARY

The fragments α' and D, isolated and purified from pepsin-digested, denatured collagen, have been characterized by molecular weight and amino acid composition. The mol. wts. are about 61 000 and 29 000, respectively. It is suggested that fragment α' is located in the B end of the tropocollagen molecule and band D material in the A end.

INTRODUCTION

In the previous paper[1] the purification of two large fragments, designated α' and band D material[2], from pepsin-digested rat-tail-tendon collagen was described. The purpose of the present work was to characterize these fragments and locate their positions in the tropocollagen macromolecule. A preliminary abstract has appeared[3].

EXPERIMENTAL

Degradation of collagen

The treatment with pepsin (EC 3.4.4.1) has been explained in detail previously[1,2].

The hydrolysis with trypsin (EC 3.4.4.4) for the fingerprints was carried out as described by LEHTONEN, NÄNTÖ AND KULONEN[4]. The protein samples were dissolved in 0.2 M ammonium bicarbonate with heating on a boiling-water bath for 5 min. After the hydrolysis, pH was brought to 4–5 with acetic acid and the samples kept on boiling-water bath for a further 5 min and then centrifuged clear. The salts were removed with Dowex-50 ion-exchange column[5].

The degradation with cyanogen bromide was carried out according to the method of BORNSTEIN AND PIEZ[6].

Estimation of the molecular weight

The sedimentation analysis was carried out with Spinco Model E analytical ultracentrifuge at two concentrations in acetate buffer, pH 4.8, I, 0.15 (ref. 7), temper-

ature, 37.7–39.6°, using 12-mm, 2° aluminium centrepieces in the analytical rotor D which was spun at 59 780 rev./min; pictures were taken at 50° phase angle. The calculations were performed according to the procedure of WILLIAMS, SAUNDERS AND CICIRELLI[8], assuming 95 000 as the weight of the α component.

Attempts were made to assess the mol. wts. from the migration rates at different gel concentrations in starch-gel electrophoresis by the method of SMITHIES[9] and from the migration rates in various Sephadex columns[10,11].

Analytical methods

Amino acids were determined with an amino acid analyser built according to the description of SPACKMAN, STEIN AND MOORE[12]. The standard deviation in the analysis of gelatin was at 570 nm $\pm 3.2\%$ and at 440 nm $\pm 5.2\%$.

Hydroxyproline was estimated according to the procedure of WOESSNER[13].

N-terminal amino acids were analysed as the DNP- derivatives applying gas chromatography[14].

To obtain the best resolution in the fingerprinting, the trypsin-hydrolysed material (corresponding to 1.25 mg protein) was applied on Whatman 3MM paper 5–6 cm from the midline on the cathode side. A 0.1 M pyridine–acetate buffer of pH 4.4 and a potential gradient of 40 V/cm were used in the high voltage electrophoresis apparatus built according to the directions of MICHL[15]. The coolant was "Varsol" (Shell). The solvent in the descending chromatography was pyridine–isoamylalcohol–water (30:30:35, by vol.)[16]. The peptides were stained with 0.2% ninhydrin in acetone.

The starch-gel electrophoresis has been described elsewhere[17].

RESULTS AND DISCUSSION

Molecular size

The calculations of the mol. wts. were based both on sedimentation studies on the fractions obtained with Sephadex G-200 column and amino acid compositions of the purified materials (Table I). Mol. wts. of 61 500 for fragment α' and 29 000 for band D material are deduced. From the sedimentation studies we estimate the mol. wt. of the band E material[2] to be about 42 000.

Attempts were made to confirm these data by electrophoresis in starch gels of varying concentrations[9] and from the migration rates in various Sephadex columns[10,11].

TABLE I

THE MOLECULAR WEIGHTS OF THE PURIFIED COLLAGEN FRACTIONS

Method	*Fraction α′*		*Band D material*	
	Mol. wt.	$s^0_{20,w}$ *(S)*	*Mol. wt.*	$s^0_{20,w}$ *(S)*
Sedimentation	62 500	2.68	28 200	1.95
	61 500	2.66		
Amino acid composition	60 500*		29 900**	
N-terminal amino groups	59 000			

* Assuming 1 histidine and 2 hydroxylysine residues.
** Assuming 1 methionine, 2 histidine and 1 hydroxylysine residues.

Neither method seems applicable to collagen fragments, because both yielded impossibly high values. When serum albumin was used as a reference, it was found that the collagen fragments emerged from the Sephadex G-200 column very early (K_d for fragment α' was 0.2 and for band D material, 0.5, but for serum albumin, 0.6)[1], presumably because their hydrodynamic volume is high because of molecular asymmetry or loose packing of the peptide chains.

A rough confirmation of the values presented in Table I was obtained from the electron microscopy of the renatured segment-long-spacing particles of the fragments (Fig. 3). The morphological work also demonstrated that the preparations were not quite homogeneous, which is reflected in the N-terminal amino acids. In fragment α', DNP-aspartic acid occupies one third of the recovered N-terminal amino acids but there are also several other N-terminal amino acids. The mol. wt. of fragment α', estimated from total DNP-amino acid content, was 59 000, which is in agreement with the sedimentation and amino acid analysis.

Amino acid composition

Table II shows the amino acid composition of the two fragments. Band D material is more basic than fragment α' and the imino acid residues are proportionally more hydroxylated than in the fragment α'. Several amino acids occur as single residues which could serve as markers in further degradation work.

TABLE II

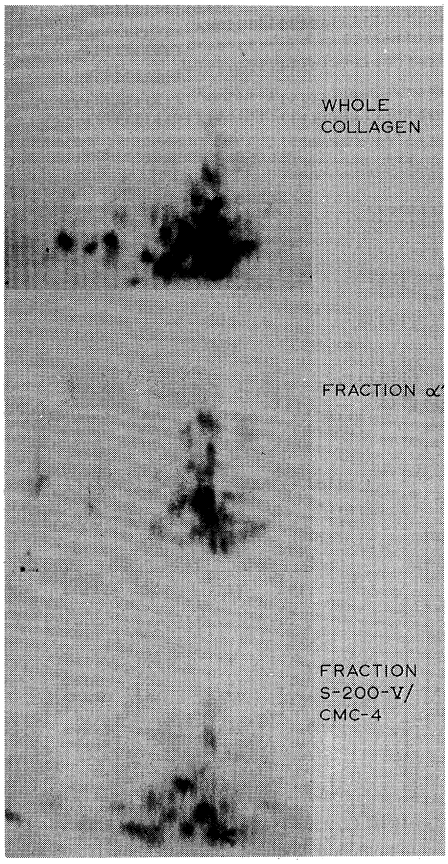
AMINO ACID COMPOSITION OF THE PURIFIED COLLAGEN FRACTIONS

Amino acid	*Residues per 1000*			*Number of residues*	
	Fragment α'	*Band D material*	*Rat collagen*[18]	*Fragment α'*	*Band D material*
Hydroxyproline	91	100	93	62	32
Aspartic acid	46	37	45	31	12
Threonine	21	20	20	14	6
Serine	34	30	43	23	10
Glutamic acid	71	54	71	49	18
Proline	128	103	121	86	34
Glycine	340	326	331	228	107
Alanine	120	109	106	80	36
Valine	21	50	24	14	16
Methionine	2	2.9	8	2	1
Isoleucine	8.3	13	10	6	4
Leucine	19	44	24	13	14
Tyrosine	0	0	3	0	0
Phenylalanine	11	11	12	7	3
Hydroxylysine	3	3.2	7	2	1
Ornithine	trace	0	0	0	0
Lysine	33	18	27	21	6
Histidine	1	6.1	5	1	2
Arginine	51	73	50	33	24
Acid residues	117	91	116	—	—
Basic residues	88	100	89	—	—
Hydroxy amino acids	149	153	166	—	—
Imino acids	219	203	214	—	—
Hydroxyproline/proline	0.71	0.97	0.77	—	—

Because it was considered that band D material may be a part of fragment α', fingerprints were prepared from whole collagen, fragment α' and band D material (Fig. 1). Band D material cannot be included in fragment α', but an overlapping is possible.

Further degradation

Fragment α' was degraded further with cyanogen bromide and pepsin (Fig. 2). No band corresponding to the D band is liberated by pepsin from the fragment α', at least not in 4 h. Three fragments corresponding to the two methionine residues were obtained with cyanogen bromide, but their separation with carboxymethyl cellulose column chromatography was not successful. Only two early-emerging peaks were observed followed by a broad bulk peak. In the sedimentation pattern of the cyanogen bromide-degraded fragment α' only one broad peak was observed with $s^{\circ}_{20,w}$ about 1.92 S.



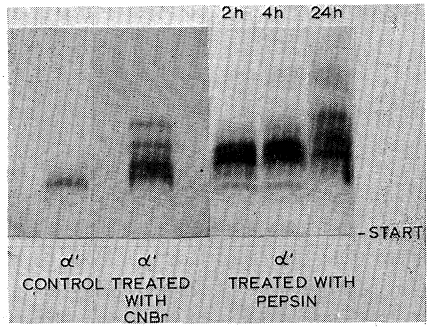


Fig. 1. Fingerprints of the trypsin-liberated peptides for the comparison of fragment α', band D material (represented by the Fraction S-200-V/CMC-4)[1], and whole collagen.

Fig. 2. Starch-gel electrophoretic patterns of fragment α', degraded with cyanogen bromide and with continued pepsin treatment.

Electron microscopy

It has been possible to renature and prepare the segment-long-spacing particles of fragment α' and band D material[19]. The electron microscopic pictures reveal without doubt that fragment α' is located in the B end and band D material in the A end of the tropocollagen molecule (Fig. 3).

General remarks

Considering the degradation of the various α components, we think that the primary action of pepsin cleaves the tropocollagen at some of the polar regions which are similarly spaced in all the components[20]. Thus similar primary fragments would arise from all the α components. However, the stability of the fragments towards

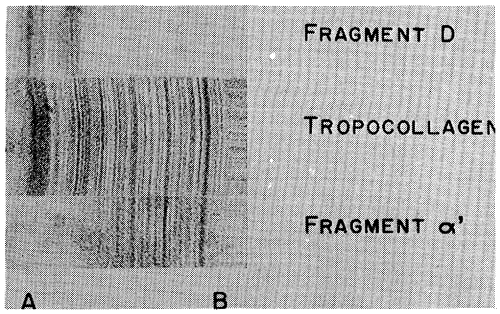


Fig. 3. Renatured segment-long-spacing particles from fragment α' and band D material compared with intact tropocollagen.

continued action of pepsin may differ according to the varying amino acid sequences.

The denaturation temperatures of both fragment α' and band D material were in the range of 27–29°.

From the calculations of the amino acid residues and from electron microscopy, it is evident that between fragments α' and band D material there remains a short gap of about 250–300 Å in the tropocollagen macromolecule.

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REFERENCES

1 K. LAMPIAHO, J. NIINIKOSKI, A. KARI AND E. KULONEN, *Biochim. Biophys. Acta*, 160 (1968) 77.
2 R. PENTTINEN, A. KARI AND E. KULONEN, *Acta Chem. Scand.*, 20 (1966) 1304.
3 K. LAMPIAHO, A. KARI, T. HOLLMÉN, J. PIKKARAINEN AND E. KULONEN, *Biochem. J.*, 104 (1967) 21P.
4 A. LEHTONEN, V. NÄNTÖ AND E. KULONEN, *Acta Pathol. Microbiol. Scand.*, 62 (1964) 141.
5 O. R. JAGENBURG, *Scand. J. Clin. Lab. Invest. Suppl.*, 43 (1959).
6 P. BORNSTEIN AND K. A. PIEZ, *Science*, 148 (1965) 1353.
7 K. A. PIEZ, E. WEISS AND M. S. LEWIS, *J. Biol. Chem.*, 235 (1960) 1987.
8 J. W. WILLIAMS, W. M. SAUNDERS AND J. S. CICIRELLI, *J. Phys. Chem.*, 58 (1954) 774.
9 O. SMITHIES, *Arch. Biochem. Biophys. Suppl.*, 1 (1962) 125.
10 P. ANDREWS, *Biochem. J.*, 96 (1965) 595.
11 A. A. LEACH AND P. C. O'SHEA, *J. Chromatog.*, 17 (1965) 245.
12 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
13 J. F. WOESSNER, JR., *Arch. Biochem. Biophys.*, 93 (1961) 440.
14 N. IKEKAWA, O. HOSHINO, R. WATANUKI, H. ORIMO, T. FUJITA AND M. YOSHIKAWA, *Anal. Biochem.*, 17 (1966) 16.
15 H. MICHL, *Monatsh. Chem.*, 82 (1951) 489.
16 C. BAGLIONI, *Biochim. Biophys. Acta*, 48 (1961) 392.
17 V. NÄNTÖ, J. PIKKARAINEN AND E. KULONEN, *J. Am. Leather Chemists' Assoc.*, 60 (1965) 63.
18 A. VEIS AND R. J. SCHLUETER, *Biochemistry*, 3 (1964) 1650.
19 K. KÜHN AND B. ZIMMERMANN, *Arch. Biochem. Biophys.*, 109 (1965) 534.
20 K. KÜHN, C. TKOCZ, B. ZIMMERMANN AND W. GRASSMANN, *Nature*, 208 (1965) 685.